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INTRODUCTION

Our overall objective is to identify molecular elements that enable breast cancer cells to establish metastatic growths. Finding rationale approaches to inhibit rate-limiting events of the metastatic growth is preferable to using systemic therapeutics that are cytotoxic on a systemic level. Cadherins make up a family of adhesion molecules that mediate Ca2+-dependent cell-cell adhesion at points of cell-cell adhesion [1]. Epithelial-cadherin (E-cadherin), the prototype classical cadherin present on the surface of most epithelial cells, has a cytoplasmic domain that anchors the cell adhesion molecule to the actin cytoskeleton via catenin-based complexes. It is generally considered that E-cadherin directs homotypic binding, organizing cells of the same lineage into a functional tissue during morphogenesis [2]. Thus, E-cadherin is central to epithelial cell differentiation and suppression of proliferation and migration.

Finding E-cadherin downregulated or even lost in invasive and metastatic carcinomas buttressed this role of E-cadherin in modulating the epithelial phenotype [3]. It has been hypothesized that loss of E-cadherin allows individual tumor cells to break from the primary tumor mass at the same time as enabling autocrine pro-proliferative and –migratory signaling to ensue from receptors and ligands physiologically separated by cell polarity and the E-cadherin-based tight junctions. This was supported experimentally when poorly differentiated and invasive carcinoma cells could be made less so by transfection with E-cadherin cDNA, with well-differentiated carcinomas becoming more aggressive when antibodies blocked. This supported a designation as a tumor suppressor, even placing E-cadherin at the apex of a "tumor suppressor system" [4]. More recent reports of E-cadherin being expressed at the site of metastatic foci in the liver, lung and lymph nodes [5] have caused reconsideration of E-cadherin downregulation as required for tumor dissemination. The key question is whether downregulation of E-cadherin is not required for dissemination, or rather, as we posit here, that E-cadherin expression and functionality is re-established at the metastatic site. *Our central hypothesis tests whether E-cadherin is necessary for cohesion between invasive breast cancer cells and the target hepatic parenchyma and that the formation of E-cadherin and connexin foci is a major rate limiting step in establishing metastatic disease.* In our second 12 months of DoD funding, we have made progress in suring the foundation of our hypothesis and confirming its relevancy to metastatic breast cancer pathologies in general; we are actively moving our *in vitro* results into a robust *ex vivo* bioreactor system, which will allow greater experimental manipulation while maintaining *in vivo* significance.

BODY

The Statement of Work (Table 1) described two tasks to effectively test our hypothesis. We have tackled the tasks in the order of greatest importance, while keeping to the schedule set forth in the original proposal. In the second year of funding, we performed important experiments that would allow us to move our experiments into the *ex vivo* system. My continued progress in the second year has made it possible to solidly move our experimental plan into a 3-D perfused liver bioreactor that will generate results fundamental to supporting our hypothesis.

Table 1. Statement of Work

Task 1A. examine the single cell architecture of breast cancer cells interactions with hepatocytes by microscopy – *completed*

Task 1B. determine the strength of the interactions using a centrifugal assay – *completed*

Task 2A. monitor protein localization using fluorescently-tagged E-cadherin – *completed (attempted real-time localization but not successful; evaluated by fluorescent microscopy)*
Task 2B. probe connexon unit integrity and transference – *future work*
Task 2C. assess the organ relevance of the cohesive interactions using an ex vivo liver bioreactor system – *in progress*

Task 1.A. *Examine the single-cell architecture of breast cancer cell's interaction with hepatocytes by microscopy.* This task is completed for 2D culture conditions. We have been successful in capturing the interaction between breast cancer cells and hepatocytes. Using human MCF7 breast cancer cells and freshly isolated rat hepatocytes, we co-cultured the cells together for 90 minutes. We observed that actin localizes to points of juxtaposition between breast cancer cells and hepatocytes (Figure 1A); further, we observed that Arp2/3, the best understood molecular determinant for actin polymerization, co-localizes with E-cadherin plaques directly juxtaposed to hepatocytes (Figure 1B). Together, these data suggest that breast cancer cells are actively anchoring themselves to hepatocytes via E-cadherin.

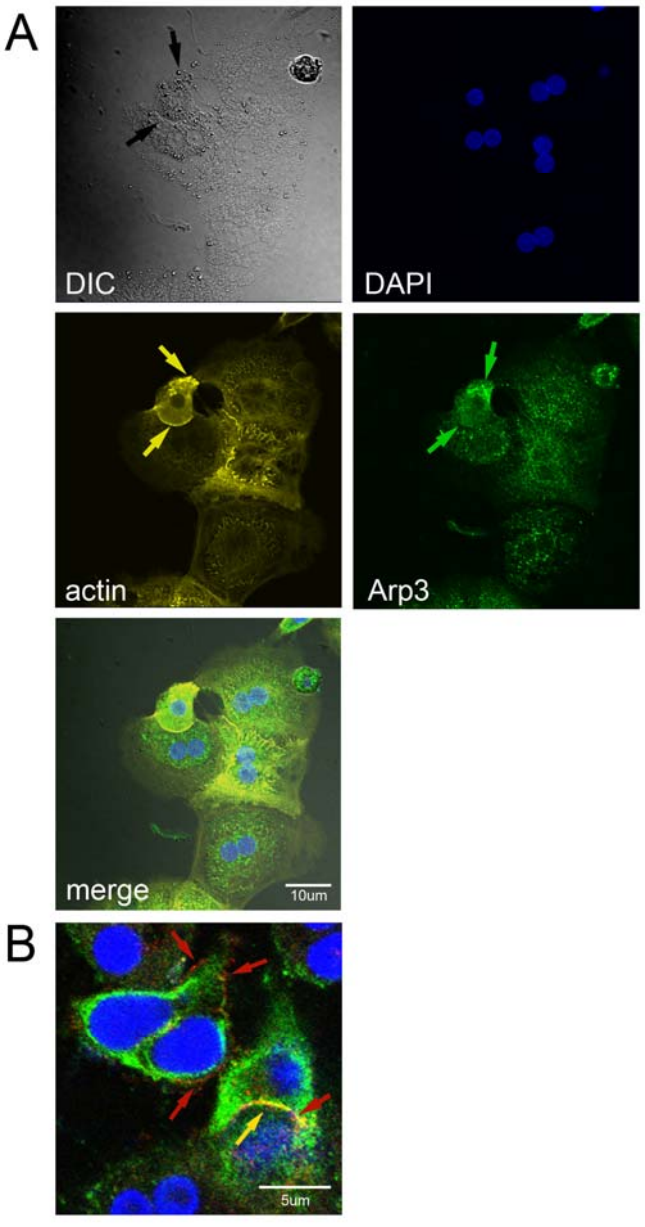

Figure 1. (A) The DIC frame shows a breast cancer cell interacting with hepatocytes. The well-differentiated multi-nucelated hepatocytes can be distinguished from the mono-nucleated cancer cell in the DAPI inset. Actin co-localizes with Arp3 at the juxtaposed membranes. (B) Arp3 also co-localizes with E-cadherin plaques on the membranes of breast cancer cells interacting with rat hepatocytes. Human-specific E-cadherin antibody (red), pan-species Arp3 antibody (green).

Task 1.B. *Determine the strength of the interactions using a centrifugal assay*. <u>This task is completed</u>. During this first year, we were able to optimize a centrifugal assay [6, 7] to study the adhesion between breast cancer cells and hepatocytes. We found that E-cadherin positive MCF7 breast cancer cells are able to form stable adhesions with hepatocytes in a similar manner to their ability to form stable adhesions with themselves. E-cadherin-negative MDA-231 cells do not form stable adhesions with hepatocytes. Further, if we disable the E-cadherin adhesion mechanism using calcium chelation or a function blocking antibody, we are able to abrogate the cohesion of the MCF7 cells to near background levels. An siRNA construct directed to E-cadherin, which knocks-down the protein significantly, also abrogates cohesion with the hepatocytes (see Figure 2).

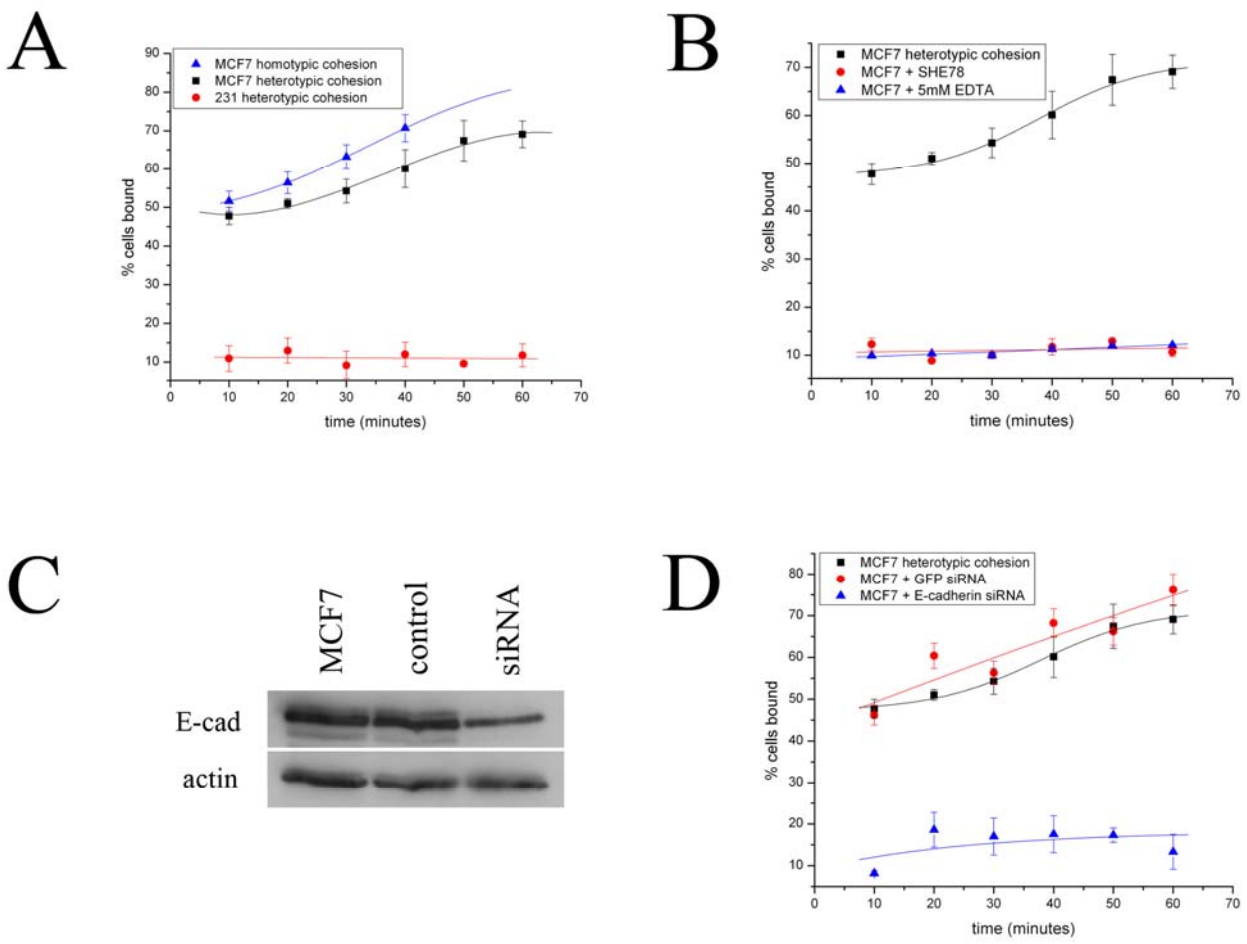

Figure 2. (A) Homotypic cohesion between MCF7-MCF7 populations occurs very similarly to heterotypic cohesion between MCF7-hepatocyte populations. MDA-231 cells do not effectively adhere to the hepatocyte population. (B) Use of calcium chelation or an E-cadherin function blocking antibody abrogates cohesion to near background levels. (C) An siRNA E-cadherin construct knocks-down E-cadherin in MCF7 to <30% of endogenous levels. (D) MCF7 cells transfected with E-cadherin siRNA adhere minimally to the hepatocyte population, while the siRNA control cells adhere similarly with the untreated MCF7 cells.

Task 2.A. *Monitor protein localization using fluorescently-tagged E-cadherin.* Our attempt at monitoring the E-cadherin mediated heterocellular adhesion mechanism was not successful. The confluency of the hepatocyte cultures and the hepatocyte autofluorescence made it difficult to evaluate which cell types were in contact using the real-time evaluation methodologies. As this plaque formation has been shown in real time by Adams *et al* [8], it was decided our research would be better focused on our other aims.

Task 2.C. *Assess the organ relevance of the cohesive interactions using an ex vivo liver bioreactor system.* Efforts are currently underway to monitor the E-cadherin interaction in two iterations of the bioreactor model: one which is compatible with real-time microscopy and another which is amenable with high-throughput terminal endpoint analysis. These studies are currently underway to look at the cancer cell-hepatocyte architecture in a robust *ex vivo* system

Supplemental Data:

Determine whether E-cadherin binding between breast cancer cells and hepatocytes initiate survival signals in the tumor cells.

During the course of the experiments, it became obvious that upon E-cadherin ligation between cancer cells and hepatocytes that canonical pathway signaling could occur. This unexpected tumor cell interaction with its metastatic micro-environment is postulated to underlie the phenomenon of chemo-resistance of breast cancer mestastases even when the primary lesion responds to chemotherapy. We examined whether breast carcinoma cell interactions with hepatocytes elicited the canonical survival pathways (ERK MAP kinase and Akt/PKB) in the breast cancer cells. Indeed, these connections activated a sustained Erk-MAPK and Akt/PKB signal (Fiugure 3).

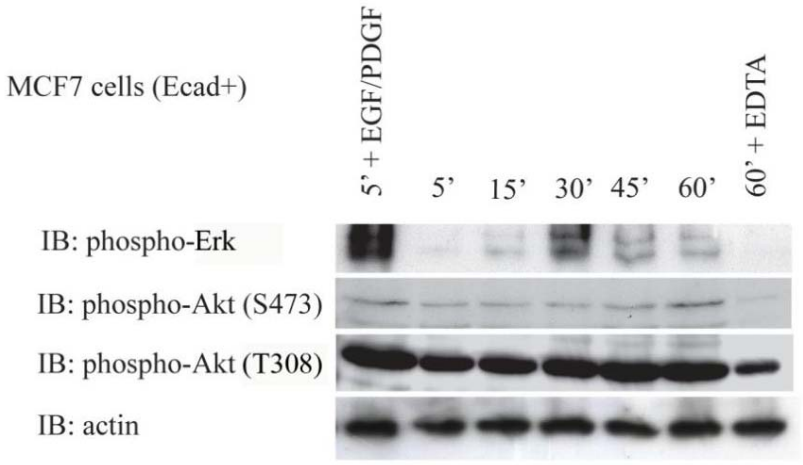

Figure 3. The Erk-MAPK and Akt pathways are activated in E-cadherin positive MCF7 cells upon ligation with hepatocyte E-cadherin. E-cadherin positive MCF7 breast cancer cells were seeded onto culture plates decorated with hepatocyte membranes. Erk activation peaks at 30' after ligation and Akt activation peaks at 60' after contact; activation of Erk and Akt can be attenuated with calcium chelation..

Determine whether E-cadherin negative invasive cancer cells re-express E-cadherin upon coculture with hepatocytes.

All of the experiments proposed in these studies used E-cadherin positive breast cancer cells (the MCF7 cell line). In order for our hypothesis to be relevant in the setting of metastatic pathogenesis, it was necessary to determine whether cancer cells that had lost E-cadherin due to methylation of the promoter region were able to convert and re-express E-cadherin upon coculture in the hepatic microenvironment. Using two DNA methylation-silenced breast cancer cell lines, MDA-MB-231 and MDA-MB-435, we observed that upon coculture with hepatocytes, the invasive cancer cells re-expressed E-cadherin and were thus able to participate in the adhesion and signaling activities shown above (Figure 4).

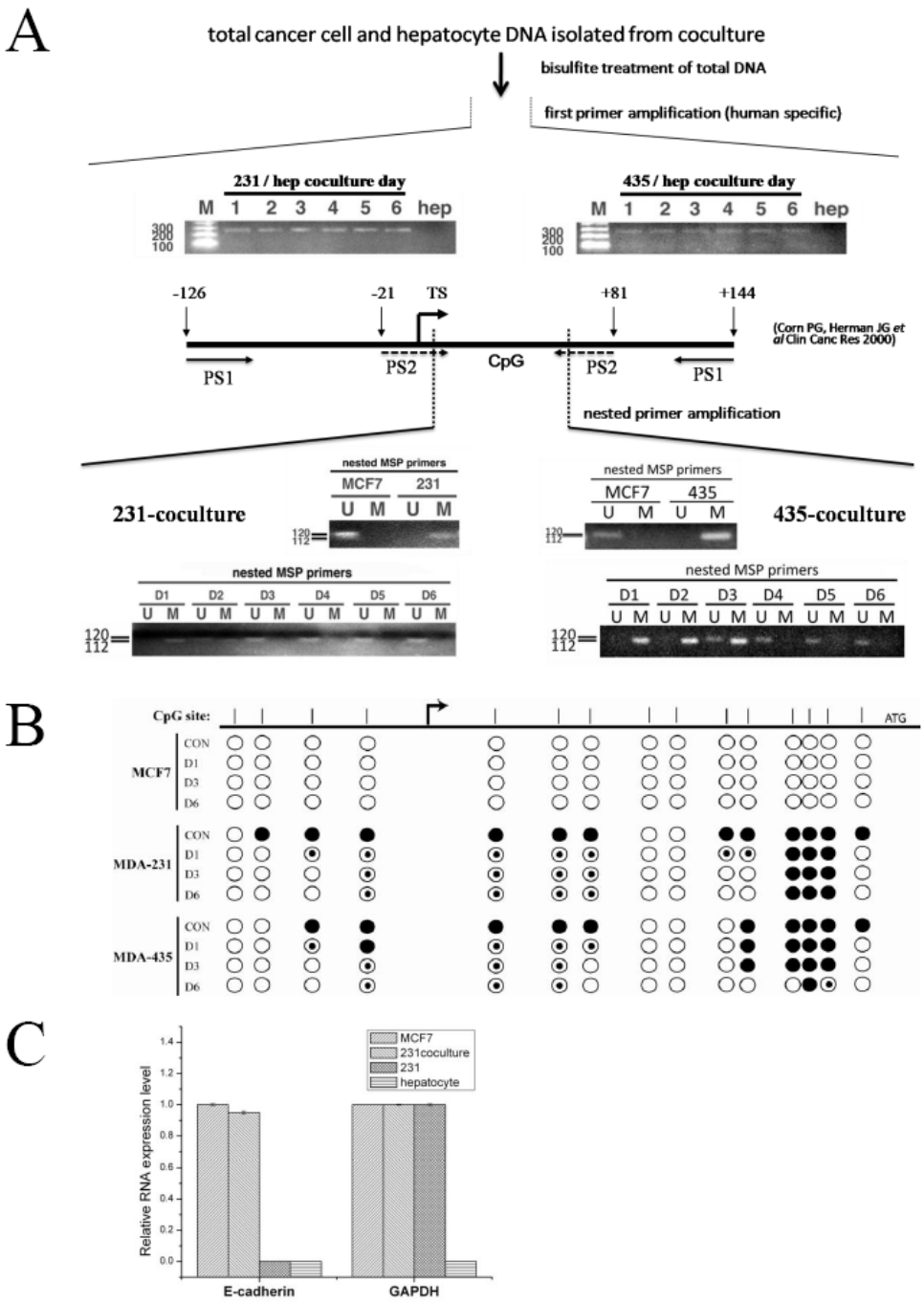

Figure 4. (A) Nested PCR method to detect methylation status of the E-cadherin promoter in a six day time course of hepatocye coculture. Above, bisulfite-treated DNA is amplified with primers that exclude CpG islands to amplify a 270bp region independent of methylation status. Below, nested primers anneal to the 270bp target to amplify a methylated (112bp) or unmethylated (120bp) fragment in the six day time course. MCF7 is used a unmethylated control. (B) Bisulfite sequencing of CpG islands in the E-cadherin promoter. Figure adapted from Corn *et al* [9]. CpG islands are indicated as vertical lines on map; each CpG island is represented a circle. MCF7, MDA-MB-231, and MDA-MB-435 were sequenced on days 1,3, and 5 coculture. Open circle, unmethylated CpG; closed circle, methylated CpG; filled circle, mixed quality values. (C) RT-PCR using human-specific primers of MDA-MB-231 cells after 6 days of co-culture with hepatocytes. Means(n=4)±s.d. Note that species specific primers do not amplify E-cadherin or GAPDH from hepatocytes.

KEY RESEARCH ACCOMPLISHMENTS

1. MCF7 cells localize actin, which functions as a cytoskeletal anchor for cell adhesion molecules, to points of juxtamembrane contact with hepatocytes.
2. MCF7 cells co-localize Arp2/3 and E-cadherin to points of juxtamembrane contact with hepatocytes.
3. Heterotypic binding between MCF7 cells and hepatocytes occurs in a single logarithmic step with kinetics similar to homotypic binding of MCF7 cells.
4. Functional heterotypic binding between MCF7 cell and hepatocytes is E-cadherin dependent and can be abrogated using calcium chelation, function blocking antibodies, and siRNA specific to E-cadherin.
5. Canonical pathway signaling occurs when E-cadherin cohesion between heptatocytes and cancer cells is initiated.
6. E-cadherin re-expression was observed in invasive breast cancer lines whose E-cadherin was epigentically silenced by promoter DNA methylation, thereby making these key research accomplishment relevant to the pathogenesis of metastatic cancer.

REPORTABLE OUTCOMES

Abstracts:

Shepard CR, A Wells. E-cadherin re-expression in breast cancer cells as a putative marker for tumor cell dormancy modeled by infiltration into hepatocyte spheroids. **Podium**; Cancer Epigenetics. Experimental Biology. San Diego, CA. 2008.

Shepard CR, A Wells. Demethylation of the E-cadherin promoter driven by hepatocytes allows of cell fate-determining signals in invasive breast cancer cells. **Podium**; Understanding Cancer for Improved Prognosis: Advances in Tumor Biology. Experimental Biology. Washington, DC. 2007.

Shepard CR, A Wells. Demethylation of the E-cadherin promoter driven by hepatocytes allows of cell fate-determining signals in invasive breast cancer cells. **Podium**; Highlights: Graduate Student Research in Pathology. Experimental Biology. Washington, DC. 2007.

Shepard CR, A Wells. Re-expression of E-cadherin by invasive breast cancer cells as a strategy for metastatic colonization of the liver. **General Session Podium**. San Antonio Breast Cancer Syposium. San Antonio, TX. 2006

Shepard CR, A Wells. Re-expression of E-cadherin by invasive breast cancer cells as a strategy for metastatic colonization of the liver. **Podium**. Biological Science Graduate Student Association Symposium. University of Pittsburgh School of Medicine. Pittsburgh, PA. 2006.

Shepard CR, A Wells. Cadherin interaction as a pathological adhesion mechanism in metastatic breast cancer. Abstract. Gordon Conference: Cell Contact and Adhesion. Andover, NH. 2005.

Reviews:

Wells A, C Yates, **CR Shepard** (2008). Mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis, in press.

Papers:

Yates C, **CR Shepard**, G Papworth, A Dash, DB Stolz, S Tannenbaum, L Griffith, A Wells (2007). Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. Adv Cancer Res *97*, 225-246.

Yates CC, **CR Shepard**, D Stolz, A Wells (2007). Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. Br J Cancer *96*, 1246-1252.

CONCLUSIONS

The second year of this three year study has reached defined milesteons and established the base for increasing productivity over the next two years of the award. The first part of the hypothesis has been repeatedly supported in the first task of the proposal. This study has also highlighted new directions concerning the signaling mechanisms that may be propogated upon heterotypic E-cadherin ligation.

Importance: The above experiments provide a 'proof of concept' that E-cadherin can participate in transformed cells *in vitro* as well as in invasive breast cancers have lost their E-cadherin due to methylation of the promoter region. These studies support that E-cadherin may be a key molecule in establishing metastatic growths in invasive cancers.

REFERENCES

1. Goodwin M, Yap AS: **Classical cadherin adhesion molecules: coordinating cell adhesion, signaling and the cytoskeleton**. *J Mol Histol* 2004, **35**(8-9):839-844.
2. Pla P, Moore R, Morali OG, Grille S, Martinozzi S, Delmas V, Larue L: **Cadherins in neural crest cell development and transformation**. *J Cell Physiol* 2001, **189**(2):121-132.
3. Hirohashi S: **Inactivation of the E-cadherin-mediated cell adhesion system in human cancers**. *Am J Pathol* 1998, **153**(2):333-339.
4. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, van Roy F: **Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role**. *Cell* 1991, **66**(1):107-119.
5. Kowalski PJ, Rubin MA, Kleer CG: **E-cadherin expression in primary carcinomas of the breast and its distant metastases**. *Breast Cancer Res* 2003, **5**(6):R217-222.
6. Giacomello E, Neumayer J, Colombatti A, Perris R: **Centrifugal assay for fluorescence-based cell adhesion adapted to the analysis of ex vivo cells and capable of determining relative binding strengths**. *Biotechniques* 1999, **26**(4):758-762, 764-756.
7. Angres B, Barth A, Nelson WJ: **Mechanism for transition from initial to stable cell-cell adhesion: kinetic analysis of E-cadherin-mediated adhesion using a quantitative adhesion assay**. *J Cell Biol* 1996, **134**(2):549-557.
8. Adams CL, Chen YT, Smith SJ, Nelson WJ: **Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein**. *J Cell Biol* 1998, **142**(4):1105-1119.
9. Corn PG, Smith BD, Ruckdeschel ES, Douglas D, Baylin SB, Herman JG: **E-cadherin expression is silenced by 5' CpG island methylation in acute leukemia**. *Clin Cancer Res* 2000, **6**(11):4243-4248.